Autolytic activation and localization in Schneider cells (S2) of calpain B from *Drosophila*

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Calpain B is one of the two calpain homologues in *Drosophila melanogaster* that are proteolytically active. We studied its activation by Ca^{2+} both *in vitro* and *in vivo*, in Schneider (S2) cells. Activation involves the autolytic cleavage, at two major sites, of the N-terminal segment, the length of which was earlier underestimated. Site-directed mutagenesis at the autolytic sites did not prevent autolysis, but only shifted its sites. Calpain B mRNA was detectable in all developmental stages of the fly. *In situ* hybridization and immunostaining showed expression in ovaries, embryo and larvae, with high abundance in larval salivary glands. In S2 cells, calpain B was mainly in the cytoplasm and upon a rise in Ca^{2+} the enzyme adhered to intracellular membranes.

Key words: autolysis, calpain, *Drosophila*, expression pattern, translocation.

INTRODUCTION

Members of the calpain superfamily pervade the Animal Kingdom, and they can also be found in lower eukaryotes (e.g. yeasts), and even in plants [1–3]. In mammals, the typical ubiquitous forms are Ca^{2+}-activated, neutral thiol proteases residing in the cytoplasm in two isoforms: μ- and m-calpain, which need micromolar and millimolar Ca^{2+} concentrations, respectively, for activation *in vitro*. Structurally, they are heterodimers consisting of a large (80 kDa) catalytic and a small (30 kDa) regulatory subunit [4,5]. Whereas large subunits for μ- and m-calpains are different, they apparently share a common small subunit that plays a chaperone-like function [6]. Recently, a second small subunit has been described [7]; its distribution and function are not known yet. Another regulator of ubiquitous calpains is the inhibitor protein calpastatin, also common in mammalian cells [8].

In mammals, at least 14 genes code for calpain superfamily proteins. Many are structurally atypical and specific in their expression in one or a few tissues [2]. Mutations in these may lead to various pathological states [9,10]. As for their mechanism of action, calpains as a rule carry out limited proteolysis on their target proteins, thereby altering their function. It is generally held that calpains are effectors in intracellular signal processing. It looks a formidable task to chart out all ramifications of calpain action.

It was workers in our laboratory who discovered calpains in the fruit fly *Drosophila melanogaster* [11,12]. A partial clone with sequence homology to vertebrate calpains was found serendipitously [13]; the full-length clone, coding for calpain A, was sequenced and the expression pattern of mRNA and protein in fly tissues studied. The second fly calpain homologue, calpain B, was assembled later from expressed sequence tag clones. Both were expressed in *Escherichia coli*, purified and characterized enzymologically [14]. According to the *Drosophila* genome database, there are two more genes in the fly genome that belong to the calpain superfamily: calpain C, an inactive homologue with all three active site residues mutated [15], and calpain D, also called Sol, which was characterized earlier [16] but not demonstrated to be a Ca^{2+}-activated protease.

In the present study we examined calpain B in more detail: its autolytic activation both *in vitro* and *in vivo*, its mRNA level and expression pattern from ovary to larval stages. Studying the distribution of endogenous calpain B and GFP (green fluorescent protein)-tagged overexpressed calpain B in Schneider (S2) cells, we found intriguing differences in their intracellular localization.

EXPERIMENTAL

Materials

Restriction enzymes were purchased from New England Biolabs. The pET 22b expression vector and *E. coli* BL21(DE3) cells were obtained from Novagen. Ni^{2+}-nitrilotriacetate resin was purchased from Qiagen. *Drosophila* Schneider cells and all reagents for maintaining them were purchased from Invitrogen. Calpain clones LD16770 and LD17211 were obtained from the BDGP (Berkley *Drosophila* Genome Project), Berkeley, CA, U.S.A. All other chemicals were from Sigma.

Construction of expression vectors

The CalpainB-pET22b construct encoding full-length calpain B was made by cloning the newly recognized N-terminal sequence into our previous incomplete clone [14] in frame by PCR from LD16770. The construct encoding the autolysed calpain B (AuCalpainBpET22b) was made with PCR from LD16770. Inactive calpain B (active-site mutant $Cys^{314} \rightarrow Ala^{314}$) was cloned into both the expression vector pET22b (ICalpainBpET22b) and the *Drosophila* transfection vector pRmeGFP on either the N- or C-terminus of GFP (ICalpainBC/NGFP). (Construction of the *Drosophila* transfection vector pRmeGFP was carried out through the modification of the pRm-Ha3 vector. Ha3 was replaced with the GFP coding sequence with multiple cloning sites on both ends.) The latter construct produces calpain B with a C-/N-terminal GFP under the control of a metallothionein promoter.

Abbreviations used: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; RT-PCR, reverse transcriptase PCR.

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Site-directed mutagenesis

The calpain B active-site mutant was made with the megaprimer method [17] with the following primer: 5′-CAGTAGCCAGGCTTCGCCAAG-3′. Calpain B autolysis sites were mutated with the QuikChange Site Directed Mutagenesis Kit from Stratagene following the instructions of the manufacturer. The primers were the following: 5′-CGAGTCATGCCGGTGTTCCGTCGTATGCGGGAC-3′ and 5′-GTCCCGCATACGACGGAACACCGGCATGACTCG-3′ for the first major site; 5′-GAAGGTGCCCGAGGCTGTTAACATGTTTTGG-3′ and 5′-CAAAACATGTTAACAGCCTCGGGCACCTTC-3′ for the second major site.

Expression of recombinant calpain B proteins in *E. coli*

The BL21(DE3) strain was transformed with the expression vectors using conventional techniques. Cells were grown in NZYM medium containing carbenicillin (50 μg/ml) at 37 °C, 250 rev./min, until $D_{600} = 0.5$–0.7. Expression was induced at 22 °C for 3 h by 0.05 mM IPTG (isopropyl β-D-thiogalactoside). The culture was cooled on ice and centrifuged at 3000 ***g*** for 10 min at 4 °C. Cells were suspended in 50 mM NaH_2PO_4/Na_2HPO_4, pH 7.5, 1 M NaCl, 5 mM EDTA, 1 % Tween 20, 2 % glycerol, 20 mM imidazole, 1 mM PMSF, 5 mM benzamidine and 20 mM 2-mercaptoethanol (suspension buffer), and sonicated for 6 × 15 s at 16 μm (MSE sonicator) on ice. The lysate was centrifuged at 100 000 ***g*** for 30 min at 4 °C. The supernatant was applied to a Ni^{2+}-nitrilotriacetate–agarose column after the addition of $MgCl_2$ to a final concentration of 5 mM. After washing with 10 column vol. of washing buffer (suspension buffer without EDTA), calpain was eluted with a step gradient of washing buffer containing 40, 60, 80 and finally 250 mM imidazole.

Fluorometric assay

Calpain activity was measured in a continuous fluorometric assay in a Jasco FP 777 spectrofluorometer. The cleavage of the fluorescent substrate, *N*-succinyl-Leu-Tyr-7-amino-4-methylcoumarin (Sigma), was monitored at excitation and emission wavelengths of 380 and 460 nm, respectively. Reaction mixtures were made up in a final volume of 50 μl at room temperature in calpain buffer (10 mM Hepes, pH 7.5, 150 mM NaCl and 1 mM EDTA). Calpains were added in a final concentration of 0.3 mg/ml, substrate was 1 mM. The reaction was started by the addition of $CaCl_2$.

Maintaining *Drosophila*

Flies were maintained in 50 ml flasks on the following medium: 1.5 % agar, 6.6 % cornmeal, 9 % sugar, 6.6 % yeast, 9.2 mM NaCl, 3.4 mM $CaCl_2$, 0.053 % phosphoric acid and 0.53 % propionic acid.

RNA preparation

Drosophila eggs, first-, second- and third-stage larvae, early and late pupae and adults were harvested from *Drosophila* culture. Total RNA to be used for reverse transcription and RT-PCR (reverse transcriptase PCR) was prepared with Trizol reagent (Invitrogen) according to the manufacturer's instructions and assayed by conventional methods. Two different fragments of the calpain B open reading frame (calpain B I and II) were amplified by PCR with two different sets of primers (forward, ATGTACGGCCATTGATAATTACC, and reverse, CTGGTTCTCGGGCACCTTC; forward, ATGTACGGCCATTGATAATTACC, and reverse, AGTCTAGATCCGGTCGTCGTGAGTACATC; 27 cycles). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control.

Antibody production against calpain B

Purified calpain B was used to immunize rabbits for polyclonal antibody production. The sera were used directly for immunoblotting without affinity purification. The antibody was checked to be specific for calpain B by immunoblot against recombinant calpain A and B.

RNA *in situ* hybridization

Digoxigenin-labelled DNA probe was synthesized by the Dig DNA labelling kit (Boehringer Mannheim) from purified calpain B cDNA. The hybridization was carried out as described in [18]. Hybridization signals were developed by the Dig Detection kit (Boehringer Mannheim) and visualized by interference contrast microscopy (Zeiss Axioscope II).

Detection of proteins in whole mount

Bleach-dechorionated embryos and dissected ovaries were collected in PBS (10 mM PBS/138 mM NaCl/2.7 mM KCl, pH 7.4). Ovaries were fixed for 10 min in a mixture of 1 vol. of fixation buffer (100 mM KH_2 PO_4, pH 6.8, 450 mM KCl, 150 mM NaCl and 20 mM $MgCl_2$), 3 vol. of distilled water and 2 vol. of 16 % formaldehyde. Embryos were fixed for 12 min in a mixture of 1 vol. of fixation buffer (100 mM Pipes, pH 6.8, 2 mM $MgSO_4$ and 1 mM EGTA), 1/10 vol. of 37 % formaldehyde and 1 vol. of heptane. The aqueous phase was removed and 1 vol. of methanol was added. Ovaries and devitellinized embryos were washed in phosphate-buffered Tris for 2 × 20 min and blocked for 1 h in blocking solution (PBS plus 0.1 % BSA, 0.1 % Triton X-100, 5 % normal goat serum and 0.02 % NaN_3). Ovaries and embryos were then incubated overnight with anti-calpain B rabbit primary antibody diluted 1:500 in blocking solution, followed by 4 × 30 min washes in phosphate-buffered Tris and a 1 h wash in blocking solution. Final incubation was with a fluorescein-conjugated anti-rabbit secondary antibody diluted 1:200 (Jackson ImmunoResearch Laboratories) in blocking solution for 2 h. Ovaries and embryos were washed again 4 × 30 min in phosphate-buffered Tris, mounted in 80 % glycerol and 4 % *n*-propyl-gallate. The embryos were analysed using a Zeiss Axioscope II microscope equipped with an Axiocam CCD camera.

Schneider (S2) cells

Cells were cultured in serum-free medium supplemented with 20 mM L-glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin at 24 °C. For visualizing vesicles cells were incubated with 50 μM BODIPY-3823 (Molecular Probes) for 1 min at 24 °C. For stable transfection of S2 cells the ICalpainBC/NGFP vectors were co-transfected with a Bluescript puromycin vector in the presence of Cellfectin reagent (Invitrogen) following the instructions of the manufacturer. The stable cell line was reached after five passages. The puromycin concentration was 50 μg/ml. Stably transfected cells expressing calpain B GFP were seeded on to glass coverslips in growth medium and induced with 0.7 mM $CuSO_4$ for 6 h in serum-free medium containing puromycin. The

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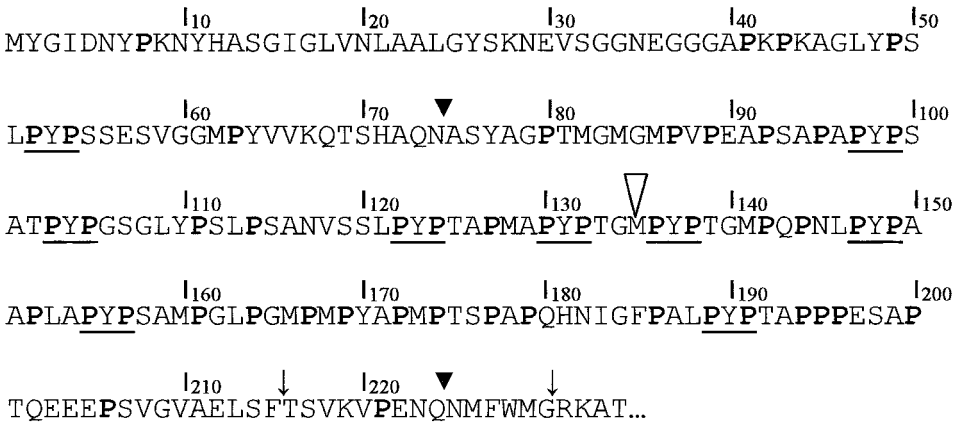

Figure 1 N-terminal sequence of *Drosophila* calpain B

The white arrowhead indicates the earlier start Met at position 135. Major autolytic sites ($N^{74}\downarrow A^{75}$ and $Q^{224}\downarrow N^{225}$) are designated by black arrowheads. Thin arrows mark autolytic sites after modification of site 224–225. Prolines are in bold, and the nine PYP motifs are underscored.

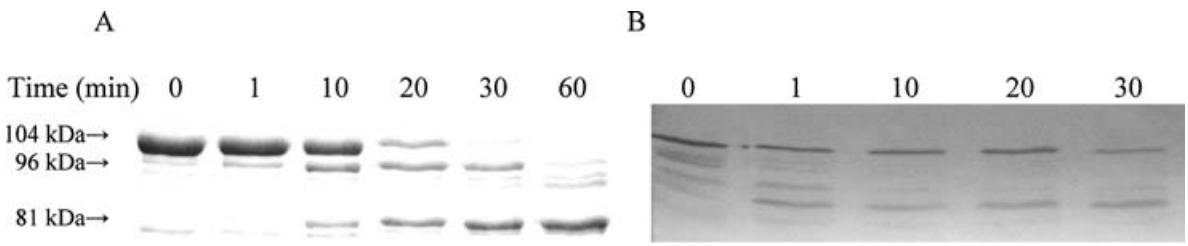

Figure 2 Autolysis of calpain B *in vitro* and *in vivo*

(**A**) Recombinant calpain B (104 kDa) was incubated with 10 mM Ca^{2+} in 10 mM Hepes, pH 7.5, for different times (indicated in min). Two major (96 and 81 kDa) and two minor autolysis products appeared; after 30 min nearly all full-length enzyme disappeared, after 60 min only the final product gave a strong signal. (**B**) Immunoblot of endogenous calpain B autolysis in S2 cells. The S2 cells were washed once in PBS, then resuspended in PBS supplemented with ionomycin to 10 μM. Incubation times are in min. All samples in the lanes were normalized to cell mass. The 0 lane is without ionomycin. Normally, calpain B and some autolysed forms are in the cells; Ca^{2+} influx promotes conversion into the final autolysis product.

induction medium was changed to PBS or PBS supplemented with 10 μM ionomycin for a maximum of 30 min. Cells were washed in PBS then fixed in 3 % paraformaldehyde for 10 min. When using antibodies, cells were permeabilized with 0.1 % Triton-X 100, blocked for 30 min in 1 % BSA, incubated for 30 min with CalpB antibody (1000× in 1 % BSA), washed three times in PBS for 2 h, incubated in secondary antibody Rhodamine Red™-X (Molecular Probes; 4 μg/ml in 1 % BSA) then washed for 2 h in PBS. Cells were mounted with MOWIOL 4–88 (Calbiochem) supplemented with DAPI and analysed by a Leica DMLS fluorescent microscope equipped with a Spot RT colour digital camera (Diagnostic Instruments).

Other methods

SDS/PAGE was carried out as described in [19]. Immunoblotting was as in [20]. For N-terminal sequence analysis samples were run on SDS/PAGE gels and blotted on to PVDF membranes (Sigma). Sequence analysis was performed using a modified-Edman-degradation sequencer program.

RESULTS

Sequence of full-length *Drosophila* calpain B

Earlier we proposed an amino acid sequence for calpain B [14] assembled from the clones available at that time. The first ATG was taken as the initiation codon, though it could not be excluded that the protein had more amino acids towards the N-terminus. As further overlapping clones (LD 16770, LD 17211) were sequenced in the *Drosophila* Genome Project, it became evident that the N-terminal segment of the protein is longer than we earlier presumed: it contains a 134 amino acid in-frame sequence upstream from the earlier starting Met (Figure 1). Thus the full length of the enzyme is 925 amino acids with a calculated molecular mass of 104 kDa. This size for calpain B was corroborated by an SDS/PAGE immunoblot run on a fresh fly extract and developed with anti-calpain B antibodies: only a single band at about 104 kDa could be seen (results not shown). The complete sequence has been deposited in the Entrez database under accession number AF062404.

Autolysis of calpain B

Stimulation of the enzyme with Ca^{2+} *in vitro*, by adding Ca^{2+} to the enzyme solution, or *in vivo*, by adding ionophore to S2 cells, results in similar autolytic conversion from 104 into 81 kDa, with intermediary fragments (Figure 2). The two major products of autolysis appear at 96 and 81 kDa. N-terminal sequencing identified the cleavage sites of the 96 and 81 kDa products: at $QN^{74}\downarrow$ASY and $NQ^{224}\downarrow$NMF, respectively (see Figure 1). The latter site is the same as found earlier with the truncated (91 kDa) calpain B [14]. The two minor fragments at about 92 and 88 kDa are much weaker, yet consistently found. Autolysis is

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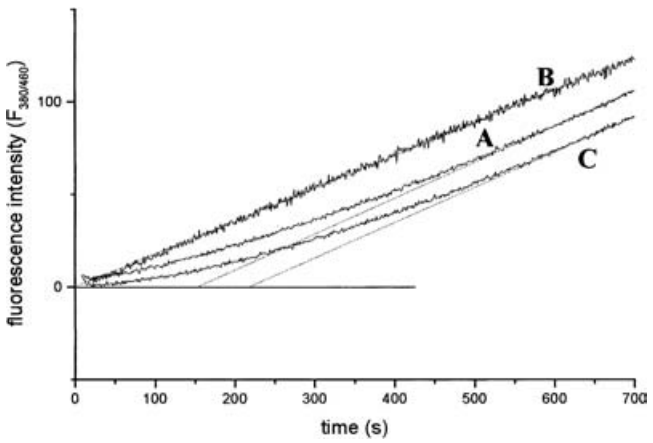

Figure 3 Activation and activity of calpain B forms *in vitro* at 10 mM Ca^{2+}

The activity of recombinant enzymes was monitored in the continuous fluorescent assay described in the Experimental section. Curve A, calpain B; curve B, autolysed (81 kDa) calpain B; curve C, autolytic site mutant calpain B. Transit time (τ) of activation was determined by fitting a straight line to the linear phase of the progress curve and extrapolating to zero substrate conversion. $\tau_A = 150$ s, $\tau_C = 220$ s.

apparently an intermolecular reaction, as in the mixture of active and inactive calpain B in a 1:10 molar ratio all enzyme protein became autolysed in the presence of Ca^{2+} (results not shown).

Activation of calpain B

Addition of Ca^{2+} induced enzyme activity in calpain B, which was continuously monitored by the cleavage of a fluorescent substrate (Figure 3). With recombinant calpain B there was a lag phase in the progress curve (transit time), to attain the linear steady-state reaction velocity later (Figure 3, curve A). In contrast, with the recombinant autolysed enzyme (calpain B Δ1–224) the progress curve was linear right from the start (Figure 3, curve B). These kinetic data suggest that intact calpain B is inactive and becomes activated during the lag phase. Further, the activation process apparently consists in the slow autolytic removal of the N-terminal segment and a fast Ca^{2+}-induced conformational change.

To check the necessity of autolysis for activation, we tried to interfere with autolysis of calpain B. Amino acids were replaced, by site-directed mutagenesis, on one or both sides of the two major autolytic sites with amino acids not accepted by calpains at the cleavage site. These amino acids were selected by a statistical analysis of calpain cleavage sites (P. Tompa, P. Buzder Lantos, A. Tantos, A. Farkas, A. Szilágyi, Z. Bánóczi, F. Hudecz and P. Friedrich, unpublished work). The following changes were made: at the first autolytic site (between N^{74} and A^{75}) the sequence $Q^{73}NA^{75}$ was substituted with GVP; at the second autolytic site (between Q^{224} and N^{225}) the sequence $N^{223}Q^{224}$ was substituted by AV (see Figure 1). These modified calpain B forms were expressed in *E. coli*, purified and tested for Ca^{2+}-induced autolysis (Figure 4). The results were unexpected: autolysis was not arrested by these mutations, but its site shifted to new, nearby peptide bonds. In the case of the site between Q^{224} and N^{225}, two new sites emerged: one at $F^{215}\downarrow T^{216}$ and one at $G^{230}\downarrow R^{231}$, as identified by N-terminal sequencing. In the case of site between N^{74} and A^{75}, modification gave rise to low-intensity, blurred band(s) which could not be analysed by sequencing. That these new sites were more sluggish than the original ones is shown by the activation time course (Figure 3, curve C): the activation lag phase with the 'autolysis mutant' was longer than with wild-type calpain B.

Calpain B expression during fly development

Total RNA was prepared from all developmental stages of the fruit fly. RT-PCR was carried out on synthesized cDNA with two different primer sets for calpain B (Figure 5). The level of calpain B message, normalized to GAPDH, was highest in the egg, which is presumably of maternal origin. In the early larval stage the level was very low, apparently as a result of degradation of maternal RNA. After this stage the level rose strongly, indicating the vigorous transcription of the CalpB gene up to the third larval stage, then the level became constant in the pupa and imago. The protein level ran almost parallel with mRNA, as detected by immunoblotting (Figure 5).

We studied the tissue expression pattern of the *CalpB* gene by *in situ* hybridization and protein immunostaining. In larval differentiation, expression varied in intensity for the different organs. Calpain B was strongly detectable in follicular and border cells of the oocyte (Figures 6A and 6B). Calpain B mRNA was evenly distributed in early embryos (Figure 6C). In late embryos, the tracheas and their orifices, as well as the larynx, stained both for mRNA (Figure 6E) and protein (Figures 6D and 6F). In the third larval stage only the salivary gland gave a strong signal for mRNA and protein (Figure 6G and 6H).

Localization of calpain B in S2 cells

We studied the localization of calpain B in Schneider (S2) cells, at resting and elevated cytoplasmic Ca^{2+} level; the latter was produced by 10 μM ionomycin. The distribution of both endogenous calpain B and calpain B overexpressed in the cells by stable transfection was examined.

Endogenous calpain B is evenly distributed in the cytoplasm, but not in the nucleus (Figure 7A). Upon a Ca^{2+} increase, the

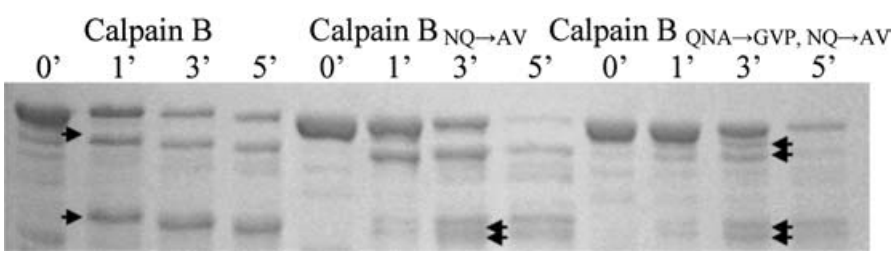

Figure 4 Change of autolytic patterns with 'autolysis mutants'

SDS/PAGE patterns of autolysis. Calpain B (control), two major autolytic fragments at 96 and 81 kDa (arrows) (see Figure 2A); calpain B ($NQ^{224} \rightarrow AV$), the mutation indicated gave rise to two new bands (double arrow) with cleavage sites at F^{215} and G^{230}; calpain B ($QNA^{75} \rightarrow GVP$, $NQ^{224} \rightarrow AV$), double mutant, both native autolysis sites shifted (double arrows). Lanes indicate changes over time (0–5 min).

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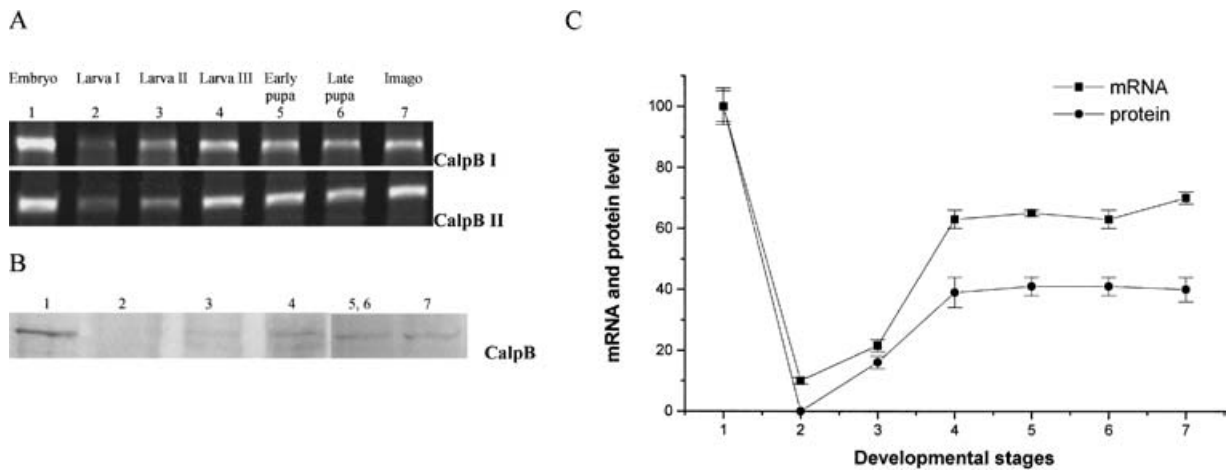

Figure 5 Calpain B mRNA and protein levels during *Drosophila* development

(**A**) RT-PCR analysis. CalpB I and CalpB II are two different fragments made by PCR (27 cycles) from two pairs of primers on the calpain B open reading frame. (**B**) Immunoblot analysis with anti-calpain B antibodies (one set out of three is shown). (**C**) Average densities of all PCR runs and immunoblots in (**A**) and (**B**), normalized to glyceraldehyde-3-phosphate dehydrogenase and α-tubulin respectively.

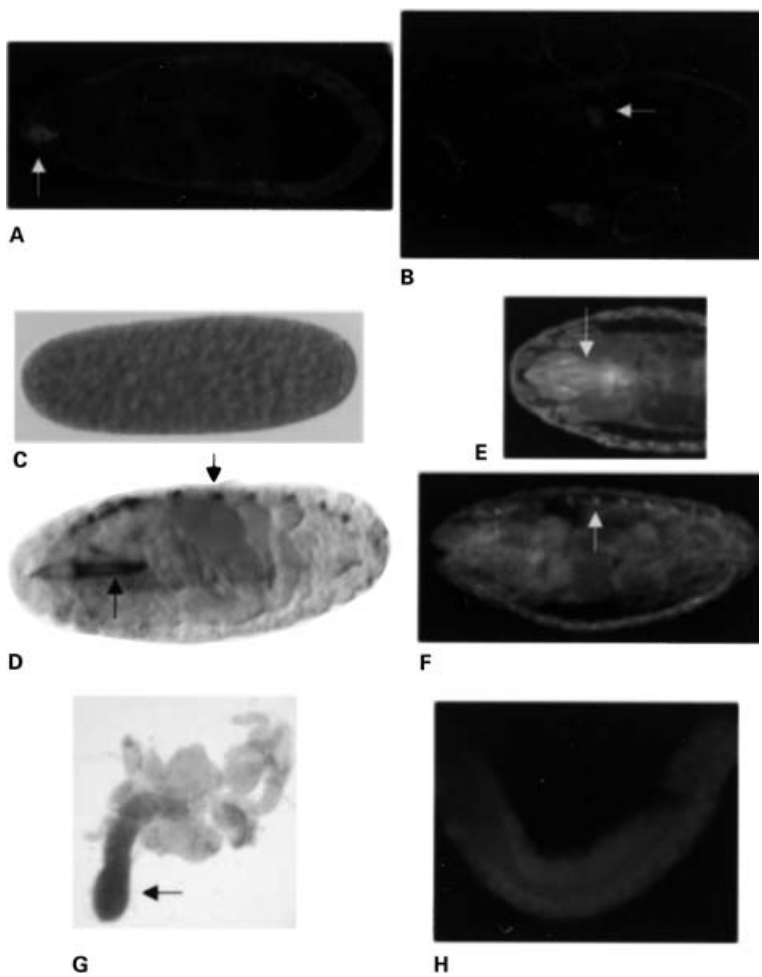

Figure 6 Expression patterns of calpain B

Calpain B mRNA and protein were detected by *in situ* hybridization and immunostaining in wild-type organs, respectively. (**A**, **B**) Immunostaining of ovaries: border cells (arrow) and follicular cells. (**C**, **D**) *In situ* hybridization of early and late embryo, respectively; calpain B mRNA is uniformly distributed in early-stage embryo (**C**) whereas it is located in the tracheal system and tongue in late-stage embryo (**D**); (**E**, **F**) immunostaining of late embryo; (**G**, **H**) calpain B mRNA and protein are localized to the salivary gland in third-stage larvae.

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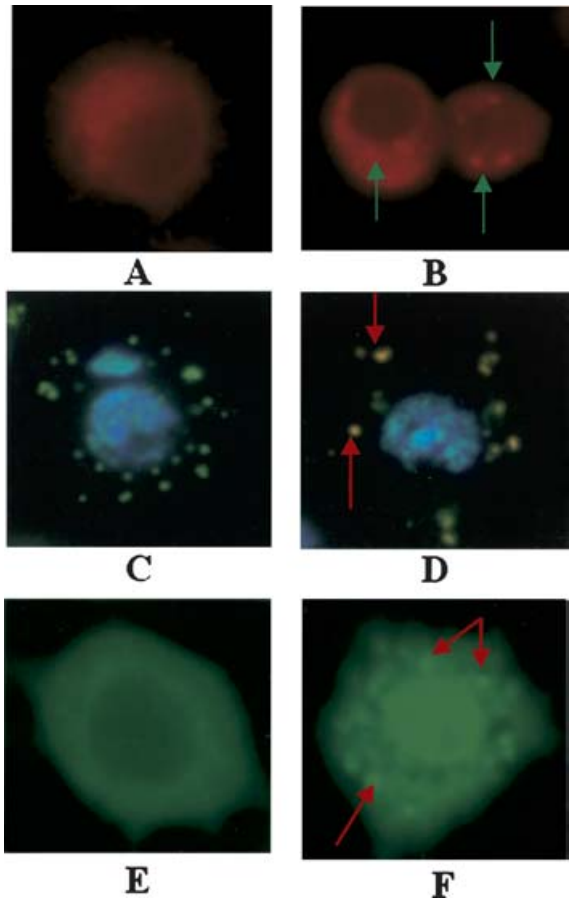

Figure 7 Localization of calpain B in S2 cells

(**A**, **B**) Immunostaining of endogenous enzyme pool with anti-calpain B, secondary antibody, Rhodamin Red-X. (**A**) Control; (**B**) cells treated with 10 μM ionomycin for 20 min. In (**A**), the cytoplasm is evenly stained, the nucleus is not stained; in (**B**), the rise in Ca^{2+} produces patches of calpain B (arrows), the nucleus remains unstained. (**C**) Nuclear (DAPI) and general membrane marker (BODIPY 500/510) immunostaining of a control cell: the nucleus is blue, vesicles are green. (**D**) Triple staining for nucleus, calpain B and vesicles, after ionomycin treatment. Vesicles that are yellow (arrows) bind calpain B, green ones do not, which points to specific membrane translocation. (**E**, **F**) GFP-fused, overexpressed calpain B without (**E**) and with (**F**) 10 μM ionomycin. In (**F**), the enzyme conjugate pervaded the nucleus, but not the nucleolus, and patchwise enzyme adherences to membranes are also discernible (arrows).

enzyme forms patches, possibly on intracellular membranous structures (Figure 7B). Indeed, calpain B immunoreactivity co-localized with some, but not all, vesicles (Figures 7C and 7D). Calpain B overexpressed in S2 cells was an inactive ($Cys^{135} \rightarrow Ala^{135}$) construct, to avoid uncontrolled autolysis and self-degradation, with GFP fused either to the N- or the C-terminal end. At low Ca^{2+} concentrations these species were found in the cytoplasm, but not in the nucleus (Figure 7E). However, upon a Ca^{2+} increase the GFP-containing enzyme forms seemed to penetrate the nucleus (Figure 7F), in addition to patch-wise adherence to internal membranes.

DISCUSSION

In this work we corrected and extended our knowledge about calpain B, which besides calpain A described earlier [13], is the other enzymically active Ca^{2+}-dependent protease in *Drosophila* resembling in domain structure the typical mammalian calpains. We turned our attention also to the behaviour of the enzyme *in vivo*, i.e. in cultured Schneider (S2) cells, a cell line derived from *Drosophila*.

The total length of calpain B was revised. Namely, completion of the *Drosophila* Genome Project revealed further sequences pertinent to calpain B: one of them added 134 amino acids to the former N-terminus [14]. Thus the length of this 'tail', from the autolytic site at residue 701, is 224 amino acids. The novel N-terminal segment has an unusual amino acid composition and sequence: it contains 22 % proline and nine repeats of the PYP motif. This segment probably has no well-defined three-dimensional structure, it belongs to the recently recognized class of intrinsically unstructured proteins [21]. As Pro-rich segments are thought to act as environmental sensors [22], we assume that this exceptionally long N-terminal tail serves, among others, interactions with various cellular components.

The relationship between Ca^{2+}-induced autolysis and activation seems clear with calpain B: activation requires autolysis. During activation of mammalian μ- and m-calpains by Ca^{2+}, autolysis occurs at the N-termini of both large and small subunits [23–25]. There is no consensus about the mechanistic role of these autolyses: some authors regard them as consequences of activation [6,26], while others hold that they are essential for activation; at least, this is strongly suggested by the close parallelism between the amount of autolysed protein and enzyme activity in the case of μ-calpain [27]. Whatever holds true for mammalian calpains, it should be remembered that their domain I is very short compared with that of calpain B. The extremely long (224 amino acids) N-terminal segment of calpain B, with nine PYP motifs and a highly flexible structure [22], may wind around the globular body of the enzyme, filling the active-site cleft and compromising activation.

With rat m-calpain the large subunit autolytic cleavage site ($Ala^{9}\downarrow Lys^{10}$), when properly mutated, failed to get autolysed, yet the enzyme was active, proving that the intact large subunit is catalytically competent [26]. With this approach we could not check whether the intact calpain B was active or not. The 'mobility' of the autolytic sites, however, indicates that the intact polypeptide chain of calpain B cannot exist in the presence of Ca^{2+}. The autolytic site 'immobility' with m-calpain as opposed to 'mobility' in calpain B, may stem from the large difference between the lengths of the two domains I: the mere 9 amino acid segment available in m-calpain may not give another chance to the enzyme.

It should be recalled that, in *Drosophila*, calpains have no small subunit and there is no calpastatin-like inhibitor. The small subunit in mammals serves a chaperone-like function in the folding of the large subunit [6,7]. Such a function is apparently not needed for calpain B or at least it is not provided by the N-terminal part, which is proved by the equal activity yields of calpain B and fully autolysed calpain B when expressed in *E. coli* (results not shown).

A putative inhibitor of calpain A and B may be calpain C, recently described by us [15]. This calpain homologue in *Drosophila* is similar to calpain A and B: in domains II and III the sequence identity with calpain B is 30 and 35 %, respectively. However, in calpain C all three active-site residues, Cys, His and Asn, have been mutated. Accordingly, this protein cannot have calpain-like protease activity. On the other hand, it may serve as a dominant-negative effector for calpain A and B, e.g. by inhibiting these via dimerization or by occupying functional binding/targeting sites in the cell.

The calpain B mRNA and protein are present all through development, with a characteristic minimum in the early larval stage. The protein is particularly abundant in oocyte development: in the follicle cells and border cell. The latter migrate from the anterior pole among the nurse cells to the border of the forming egg, thereby offering a readily monitored model of cell motility and the role of calpain(s) therein. A great number of observations

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on mammalian systems support the assumption that calpains are instrumental in the processes of cell motility and migration [28].

Calpain B indigenous in S2 cells is evenly distributed in the cytoplasm, as revealed by fluorescence microscopy. Upon a rise in cytoplasmic Ca^{2+} level, it undergoes autolysis and adheres to intracellular membranes. In S2 cells stably transfected with inactive ($Cys^{135} \rightarrow Ala^{135}$) calpain B with a GFP tag fused to the N- or C-terminus, the overexpressed enzyme is again in the cytoplasm at resting Ca^{2+} level. When Ca^{2+} becomes elevated, however, the enzyme conjugates appear to enter the nucleus. This intriguing observation may be due to the GFP-tagged large enzyme pool produced by overexpression. GFP has been successfully used in a great number of studies in which it apparently did not influence the behaviour of the carrier protein [29]. On the other hand, GFP is highly soluble [30], thus it may favour aqueous niches, such as the flexible and dilatable nuclear pore channel [31]. In fact, mammalian μ-calpain was shown to undergo ATP-dependent nuclear translocation [32]. In the case of calpain B, however, nuclear translocation upon a Ca^{2+} increase seems to be an artefact which does not occur at normal cellular levels of the untagged enzyme.

Understanding the organization and functions of the *Drosophila* calpain system will pave the way to solving the more demanding case of the human system.

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